HUMAN BLOOD GROUP B TRISACCHARIDE. SYNTHESIS, CHARACTERIZATION, AND USE IN THE GENERATION AND SELECTION OF MONOCLONAL ANTIBODIES WITH A KNOWN SPECIFICITY.(*)

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SUMMARY

The trisaccharide specific for human blood group B was obtained as a glycoside of 8-hydroxy-3,6-dioxaoctanal. A new galactose intermediate was developed for chain extension at 0-2 or 0-3 in either sequence. The use of trichloroacetimidates as glycosyl donors for the establishment of the two α-glycosidic linkages was also noteworthy. Human blood group B trisaccharide coupled to KLH was used to induce high anti-B titer in balb-c mice for the production of anti-B monoclonal antibodies. The hybridomas were selected by their reaction with the trisaccharide and by their specific agglutination of B erythrocytes. The monoclonal antibody LAGS-B-03 thus selected displayed excellent parameters as a blood-typing reagent.

RESUMEN

Un trisacárido específico del grupo sanguíneo humano B fue obtenido en forma de glicósido del 8-hidroxi-3,6-dioxaoctanal. La síntesis contempla el uso del método de los tricloroacetimidatos para establecer los dos enlaces α y un nuevo intermediario de la galactosa que puede ser extendido indistintamente por las posiciones 2 y 3. El trisacárido del grupo sanguíneo humano B acoplado a KLH se empleó para inducir alto título anti-B en ratones balb-c para la producción de anticuerpos monoclonales anti-B. Los hibridomas fueron seleccionados sobre la base de su reacción con el trisacárido y luego por su capacidad de aglutinar específicamente los eritrocitos B. El anticuerpo LAGS-B-03 seleccionado demostró excelentes parámetros como reactivo hemoclasificador.

INTRODUCTION

The synthesis of oligosaccharides and its use in the preparation of monoclonal antibodies with a known molecular specificity is one of our main research goals. This strategy for the preparation of blood-typing reagents (1) or monoclonal antibodies against tumor-associated antigens (2) is well documented. Monoclonal antibodies are displacing conventional polyclonal reagents in routine uses as red-cell grouping reagents mainly because in this way better reagents can usually be obtained and continuing the hyperimmunization of human donors thus becomes unethical.

In the '80s the human ABO blood group system was extensively studied and many good specific monoclonal antibodies were obtained. Experience accumulated during these years and the requirements of our blood banks prompted us to choose the human blood group B trisaccharide as our first model.

A good monoclonal anti-B antibody should display not only a fine specificity for the trisaccharide that is the epitope defining human blood group B, but also should agglutinate B red cells with a given avidity and affinity.

The following scheme was intended to prepare efficiently monoclonal antibodies fulfilling all these parameters:

- Synthesis of the B trisaccharide with an appropriate bifunctional molecule (spacer).
- Coupling of the synthetic trisaccharide with two different proteins; the first is as highly immunogenic carrier for immunization, the second as inert carrier for estimating of the anti-B specificity.
- Immunization and fusion.
- Selection of cell lines producing the desired antibodies by their fine molecular specificity for B trisaccharide, good avidity and affinity showed in the B erythrocyte agglutination.

In the present paper, we outline the preparation of a monoclonal antibody LAGSB-03 with the use of this methodology.

MATERIALS AND METHODS

Synthesis

General procedures

Optical rotations were measured with a POLAMAT A automatic polarimeter for 1% chloroformic solutions at 22-24°C. ^{1}H and ^{13}C NMR spectra were obtained in a BRUKER AC-250F spectrometer. $CDCl_3$ was generally used as a solvent and tetramethylsilane as a standard δ (0.0 ppm). The assignments were accomplished

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based on homo(H-H) and hetero(X-H) nuclear COSY experiments. Thin layer chromatography was performed using precoated MERCK plates which were developed with a 10% H_2SO_4 in ethanol and charried. The solvent systems used were: A, dichloromethane-acetone; B, hexane-ethyl acetate; C, ethyl acetate-methanol-water.

All the compounds were purified for characterization by flash chromatography under low nitrogen pressure. Elemental analysis was performed in our Laboratory by the combustion method. The carbohydrate concentration of conjugates was estimated using the phenol-sulphuric acid method (3). The protein concentration was estimated by Lowry's method (4).

3-*O*-Allyl-4,6-di-*O*-benzyl-1,2-*O*-(1-methoxyethylidene)-α-D-galactopyranose (2). To a solution of acetobromogalactose **1** (15 g, 36.5 mmol) in dry CH_2Cl_2(67.5 mL) were added Et_4NBr (18.1 mmol), dry methanol (4.4mL, 109.5 mmol) and 2,4,6-trimethylpyridine (9.6 mL, 73.0 mmol). Then, the resulting solution was refluxed at 50°C for 3 hr. After the reaction was completed (TLC A; 20: 1), it was treated with cool water, saturated aqueous sodium hydrogen carbonate solution, and water. The organic layer was dried and evaporated. The resulting syrup was dissolved in methanol(150 mL) followed by the addition of 1% sodium in methanol until pH 9. The solution was stirred for 30 min, and when deacetylation was finished (system A, 1:1). After this, it was evaporated to a syrup and dried *in vacuo*.

A solution of the orthoester and dibutyltin oxide(8.46 g, 34 mmol) benzene (200 mL) was heated under reflux with continuous removal of water for 12 h. Then, Bu_4NI (12.56 g, 34.0 mmol) and allyl bromide (3.5 mL, 41.0 mmol) were added and the reflux was continued for another 5 hr, the solvent was removed *in vacuo* and the residue was dissolved in ethyl acetate (100 mL) to remove the salts that crystallized. The mother liquour was evaporated and the residue was dissolved in CH_2Cl_2 (150 mL). To this solution were added a 50% aqueous KOH solution(15 mL), Et_4NBr(23 g, 109.5mmol) and benzyl bromide (13 mL, 109.5 mmol) and the mixture was refluxed while stirring. After 24h, TLC (system B, 4:1) indicated a faster moving spot (RF 0.60). The mixture was filtered, washed with water, dried and evaporated. Column chromatography (toluene) of the residue afforded **2** (30%) as a syrup: $[\alpha]_{578}$= + 10.2°.^{1}H NMR δ(ppm) 7.3(m, 10H, Ph), 5.95 (m, 1H, -CH=), 5.73 and 5.55(d, H-1), 4.52(H-2), 4.33(H6), 4.07(H-4), 3.92(H-), 3.67(H-3), 3.35 and 3.25(s, 3H, OCH_3), 1.7 and 1.54(s, 3H, CH_3). ^{13}C NMR δ(ppm) 134.5 (-CH=), 128.0 (Ph), 122.4 (C-ortho), 116.7 (= CH_2) 97.7 and 97.0 (C-1), 80.3 (C-3), 79.8 (C-2), 74.4 (CH_2Ph), 73.5 (CH_2Ph), 73.2 (C-4), 72.8 (C-5), 70.4 (CH_2OAll), 68.0 (C-6), 50.0 and 49.5 (OCH_3), 24.5 and 23.3(CH_3). Anal. Calcd for $C_{26}H_{32}O_7$:C,68.40%; H, 7.07%. Found: C, 68.14%; H,7.08%.

4-(1,3-Dioxolane-2-yl)-3-*O*-oxabutyl 2-*O*-acetyl-4,6-di-*O*-benzyl-β-D-galactopyranoside (4). A solution of **2** (2.04 g, 4.5 mmol) in dry CH_2Cl_2 (42 mL) Et_4NBr (0.48 g, 2.3 mmol) and 4A molecular sieves (3.1 g) was stirred for 1 h. Then, acetyl bromide (0.7 mL 9.0 mmol) was added and after 10 min (TLC, system B, 4: 1) the reaction was quenched with ice-water, the organic layer was washed with aqueous saturated sodium hydrogencarbonate and water, dried and evaporated. A solution of the bromide in dry CH_2Cl_2 was added under argon to a stirred solution of the spacer (0.74 g, 5.0 mmol), mercuric (II) bromide (1.95 g, 5.4 mmol) and 4 A molecular sieves (4.1 g) in dry CH_2Cl_2. The mixture was stirred for 24 hr (TLC, system B, 3: 1), then diluted with CH_2Cl_2 (15 mL), filtered and washed succesively with a 10 % aqueous potassium iodide, aqueous sodium hydrogen carbonate and water. The organic layer was dried, evaporated and used directly for the de-allylation step.

A suspension of the residue (2.38 g)and 5 % Pd/C (2.38 g) in ethanol-acetic acid-water(4: 4: 1, 48 mL) was refluxed with stirring for 5 hr. The mixture was filtered, concentrated and co-concentrated with toluene (2 x 10 mL). Column chromatography (system B, 3: 1) of the residue afforded **4** (32%) as a syrup: $[\alpha]_{578}$= + 4.5° ^{1}H NMR δ(ppm) 7.3 (m, 10H, Ph), 5.02 (dd, H-2), 4.97 (t, 1H, CH-dioxolane), 4.69 (2H, CH_2Ph), 4.49 (2H, CH_2PH), 4.43(d, H-1, J_{1-2}= 7.9Hz), 3.9 (H-6), 3.88 (2H, CH_2-spacer), 3.81 (H-4), 3.75 (2H, CH_2-spacer), 3.68 (H-6), 3.62 (H-3), 3.63 (2H, CH_2-spacer), 3.6 (H-5), 2.1 (s, 3H, CH_3). ^{13}C NMR (ppm) 171.1 (C= O), 128 (Ph), 102.5 (CH-dioxolane), 100.8 (C-1), 76.5 (C-4), 75.3 (CH_2Ph), 73.5 (CH_2Ph), 73.3 (C-5), 73.2 (C-2), 73(C-3), 72.0(CH_2-spacer), 70.8(CH_2-spacer), 68.6 (C-6), 64.9 (CH_2-CH_2dioxolane), 20.9 (CH_3 Ac). Anal. Calcd for $C_{28}H_{36}O_{10}$ C, 63.15%; H, 6.81%. Found: C, 63.15%; H, 6.78%.

2,3,4-tri-*O*-Benzyl--L-fucopyranosyl tricloroacetimidate (6). To a solution of tribenzylfucose (0.21 g, 0.5 mmol) in dry CH_2Cl_2(6 mL) were added anhydrous K_2CO_3 (0.21 g, 1.5 mmol) and trichloracetonitrile (0.3 mL 2.5 mmol). The mixture was stirred for 20 hr at room temperature. Then, diethyl ether (5 mL) was added and the suspension was filtered over celite, evaporated and used directly for fucosylation. Yield 81 %. ^{1}H NMR (ppm)- 8.6(s 1H NH); 7.5-7.3(m 15H Ph); 5.8(d H-1); 5.6-4.7(m 6H CH_2Ph); 1.3(d 3H CH_3).

4-(1,3-Dioxolane-2-yl)-3-*O*-oxabutyl 2-*O*-acetyl-4,6-di -*O*-benzyl-3-O-(2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl)-β-D-galac topyranoside (7). A solution of **4** (0.43 g, 0.9 mmol) and **5** (0.62 g, 0.9 mmol) in anhydrous diethyl ether (13 mL) was stirred for 20 min in the presence of 4 A molecular sieves under a nitrogen atmosphere. Then, trimethylsilyl trifluoromethanesulfonate (0.163 mL, 9 mmol) was added slowly. After 20 h, the reaction was quenched by adding triethylamine, then diluted with CH_2Cl_2, filtered and evaporated. Column chromatography (system B, 6:1) of the residue afforded **7** (69 %) as a syrup: $[\alpha]_{578}$= + 38.6°. ^{1}H NMR (ppm) 7.3(m 30H Ph); 5.32(dd H-2 Gal'); 5.0(t CH-dioxolane); 4.95 (d H-1 Gal' J_{1-2}= 3.5Hz); 4.39(d H-1 Gal' J_{1-2}= 10.8Hz); 4.07(dd H-2 Gal'); 3.95(2H CH_2-spacer); 3.92(H-4 Gal'); 3.9(H-3 Gal'); 3.85(2H CH_2-spacer); 3.68(H-3 Gal'); 2.0(s 3H CH_3). ^{13}C NMR (ppm) 169.7(C= O); 128.0(Ph); 102.7 (CH-dioxolane); 101.5(C-1 Gal'); 99.5(C-1 Gal'); 78.9(C-3 Gal'); 76.6(C-2 Gal'); 74.9(C-4 Gal'); 73.5(CH_2Ph); 71.1(C-2 Gal'); 65.0(CH_2-CH_2 dioxolane); 21.1(CH_3 Ac). Anal.Calcd: C, 70.57%; H, 6.69%. Found: C, 70.01%; H, 7.15%.

4-(1,3-Dioxolane-2-yl)-3-*O*-oxabutyl 4,6-di-*O*-benzyl-3-*O*- (2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl)-2-*O*-(2,3,4-tri -*O*-benzyl-α-L-fucopyranosyl)-β-D-galactopyranoside (8). To a solution of **7** (0.32 g, 0.3 mmol) in dry methanol (4 mL), sodium methoxide was added to pH 9. After 45 min, TLC (system A, 10:1) revealed complete deacetylation of the starting material. The mixture was neutralized with Dowex-50 (H^+) resin, filtered, concentrated and dried *in vacuo*. To a solution of the residue, **6** (0.37 g, 0.6 mmol) and 4 A molecular sieves in anhydrous diethyl ether (15 mL) was injected trimethylsilyl trifluoromethanesulfonate (0.271 mL, 1.5 mmol) under argon. After 20 h, the reaction was quenched by adding triethylamine, then diluted with CH_2Cl_2, filtered and evaporated. Column chromatography (system B, 6: 1) of the residue afforded **8** (49 %) as a syrup: $[\alpha]_{578}$= - 10.6°. ^{1}H NMR (ppm)- 5.58(d H-2 Fuc J_{1-2}= 3.3Hz); 5.31(d H-1 Gal'); 4.37(H-5Fuc); 4.25(d H-1 Gal'); 4.07(H-2 Gal'); 4.03 (H-2 Fuc); 3.95(H-3 Gal'); 3.9(H-3 Fuc); 3.76(2H CH_2-spacer) ; 3.65(H-4 Fuc); 1.1(d 3H CH_3 Fuc). RMN-^{13}C (ppm) 128(Ph); 102.7(CH-dioxolane); 102.4(C-1 Gal'); 97.3 (C-1 Fuc);96.9(C-1 Gal'); 77.8(C-4 Fuc); 76.8(C-2 Fuc); 66.3(C-5 Fuc); 65.0 (CH_2-CH_2-dioxolane); 16.7(CH_3 Fuc). Anal. Calcld: C, 73.09%; H, 6.77%. Found: C, 73.00%; H, 6.78%.

4-(1,3-Dioxolane-2-yl)-3-*O*-oxabutyl 2-*O*-(2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-4,6-di-*O*-acetyl-β-D-galactopyranoside (9). A suspension of **8** (0.0724 g 0.03 mmol) and 5% Pd/C (0.036 g) in methanol (4 mL) was stirred under hydrogen. After 24 h, TLC (system C, 20: 20: 1) revealed complete conversion of the starting material. The mixture was filtered, evaporated and dissolved in pyridine-acetic anhydride (2:1, 2 mL), the solution was stirred for 48 hr, concentrated and co-concentrated with toluene (2 x 10 mL) to afford **9**: ^{1}H NMR δ(ppm) 5.57(H-4 Gal'), 5.51 (d H-1 Fuc), 5.4(H-3 Gal'), 5.39(H-4 Gal'), 5.39(H-1 Gal'), 5.28(H-2 Gal'), 5.25(H-4 Fuc), 5.21(H-2 Fuc), 5.09(H-3 Fuc), 5.05 (H-dioxolane), 4.48(H-5 Gal'), 4.45(H-1 Gal'), 4.42(H-5 Fuc), 3.85(H-3 Gal'), 3.8(H-5 Gal'), 3.68(H-2 Gal'), 1.15(d 3H CH_3 Fuc). ^{13}C NMR (ppm) 170.0(C= O), 103.7(C-1 Gal'), 102.6(CH-dioxolane), 97.5(C-1 Fuc), 92.5(C-1 Gal'), 76.9(C-2 Gal'), 73.1(C-3 Gal'), 71.9(CH_2-spacer), 71.2(C-4 Fuc), 70.7(CH_2-spacer), 70.4(C-5 Gal'), 69.6(CH_2-spacer), 68.2(C-2 Fuc), 68.1(C-4 Gal'), 67.6(C-3 Fuc), 67.1(C-2, C-3 Gal'), 66.6(C-5 Gal'), 65.4(C-5 Fuc), 65.0(CH_2-CH_2-spacer), 64.7(C-4 Gal'), 20.3(CH_3 Ac), 16.1(CH_3 Fuc).

Ring-opening of a dioxolane and deacetylation. A solution of **9** (0.0596 g) in formic acid (1 mL) was stirred for 2 hr at room temperature (TLC, system A, 6: 1, aniline phthalate). The acid was removed with nitrogen gas at room temperature, toluene was coevaporated several times from the residue. ^{1}H NMR 9.73(HC= O); 5.59(H-4 Gal'); 5.51(H-2 Fuc); 4.45(H-1 Gal'); 1.7(d 3H CH_3 Fuc). ^{13}C NMR (ppm) 103.8(C-1 Gal'); 97.5(C-1 Fuc); 92.5(C-1 Gal'); 70.7(CH_2-spacer); 66.6(C-5 Gal'); 65.5(C-5 Fuc); 64.6(C-4 Gal'); 16.2(CH_3 Fuc).

A solution of MeONa in anhydrous methanol (0.1 mol/L) was added at O°C to a solution of the residue in dry methanol(0.3 mL) until pH 9. After 45 min, TLC (system D, 15: 3 : 10: 12) revealed complete deacetylation of the starting material. The mixture was neutralized with acetic acid, evaporated at room temperature and used directly for conjugation.

Conjugation with proteins (6). The free trisaccharide was quantified by the phenol-sulphuric acid method, using a mixture of the three monosaccharides as standard. The trisaccharide (10 mg) was conjugated with BSA or KLH in a borate buffer (0.2 M), pH 9 at 50 °C for 36 hr using sodium cyanoborohydride according to the following molar proportion: 112/1/560 for BSA and 1000/1/5000 for KLH. The resulting solution was dyalised affording B24-BSA and B336-KLH.

Production and selection of monoclonal antibodies

Immunization

Female balb-c mice, 3 months old (CENPALAB, CUBA) were injected i.p. with a trisaccharide conjugate to KLH (40 μg/ mouse/ inoculation) emulsified in Freund complete adjuvant (0.1 mL). Reimmunization was performed with incomplete Freund adjuvant s.c. 3 times at biweekly intervals. The mice were bled 10 days after the last inoculation and sera were tested for antibodies binding to the oligosaccharides coupled to BSA by an ELISA described below. Prior to fusion of spleen cells, a booster inoculation of 200 μg antigen x mouse in saline (0.5 mg/mL) was administered i.p. and i.v. at 3, 2 and 1 days before fusion.

ELISA for antibodies binding to purified antigens. Microtiter PVC ELISA plates were coated with a solution of the corresponding oligosaccharide coupled to BSA (100 μL/well, 4 μg/ mL) in sodium hydrogencarbonate-carbonate buffer (0.1 M) pH 9.6. After incubation for 2 h at 37°C plates were washed twice with PBS and incubated for 1 h at 37°C with a blocking solution of 0.2 % of BSA (150 μL/well) and washed with phosphate buffered saline solution (PBS) plus 0.05 % Tween 20.

The hybridoma supernatants or serum dilutions of immunized mice were incubated for 2 h at 37°C, washed twice and incubated for 1 h with a rabbit anti-mouse IgG (whole molecule) conjugated with horseradish peroxidase.

After four washings, substrate ortho phenylendiamine was added and after 20 min the reaction was quenched with a 2.5 M sulphuric acid solution. Absorbtion was recorded in an ELISA plate reader (Titertek Multiskan).

Fusions. The free trisaccharide obtained from **9** (4 mg) was coupled to biotinyl hydrazide (1 mg) under mild conditions (PBS, pH 7.4). The non secreting X63-Ag 8.653 plasmacytoma cell line was maintained in Dubelcco's modified Eagle's medium (DMEM) containing 6-thioguanine and 10 % fetal calf serum. A week before fusion the cell line was grown in DMEM without 6-thioguanine. All media employed routinely contained gentamicin (40 μg/mL). Spleen cells of one immunized mouse were collected by perfusion with PBS pH 7.4. The splenocytes (10 x 10^7/mL) were incubated with biotinylated antigens (10 μg/mL) for a period of 20 min at 4°C and washed twice with PBS (30 mL) followed by centrifugation (10 min, 1000 rpm). The cells were resuspended in PBS and streptavidin (20 μg/mL) was added and incubated for 20 min at room temperature and then washed 2 times.

The mieloma cells X63Ag 8653 (10 x 10^6) were biotinylated with biotin N-hydroxisuccinimide ester. Briefly, biotin N-hydroxysuccinimide ester in DMSO (50 μg, 1 mg/mL) was added to the cells (2 mL). After 20 min at 4°C, the cells were washed two times with PBS. The splenocytes (1 x 10^8) and myeloma cells (1 x 10^6) in PBS (2 mL) were incubated for 15 min at 37°C and pelleted at 1000 rpm 10 min and supernatants discarded. Fusion of spleen cells and myeloma was performed with polyethylene glycol according to a previously described technique (7).The cloning of positive ELISA wells was performed by limiting dilutions.

Production of ascitic fluid. 2,6,10,14 tetramethylpentadecane primed mice were injected with 1 x 10^6 hybridoma cells in PBS. After 12-16 days ascitic fluid was tapped, centrifuged, filtered and stored at -20°C.

Affinity constant (8). Ascites fluid of LAGS-B-03 was purified by affinity chromatography on protein G sepharose Fast Flow (Pharmacia LKB) using a standard protocol. The affinity constant of the antibody was measured by the Beattys method (8) which is based on the law of mass action and uses total antibody concentration added per well rather than bound to free ratio. The equation K_{aff}= 1/2 (2[Ab']$_t$-[Ab]$_t$) is an estimate of the affinity constant of the antigen-antibody interaction that is based solely upon the total antibody concentration at OD .50 for plates coated with two amounts of antigens one being half the other

[Ab]$_t$= Total antibody added

[Ab']$_t$= Total antibody concentration at OD.50.

We used four different B-BSA coating concentrations, 0.25, 0.125, 0.062 and 0.031 μg/mL. For each coating solution, serial dilutions of the purified monoclonal antibody specific for B group were applied to different rows on the microtiter plate. Each row was done in duplicate and the antibody concentration at OD .50 for each pair of serial dilutions was calculated.

The antibody was tested in this fashion in three different experiments, each test being performed on a different day. The result was expressed as Kaffinity(+ - standard deviation) in mol^{-1}

Serological evaluation of mab

Freshly collected blood from healthy volunteers or donors from groups A_1, A_2, B, B cord, A_1B, A_2B and O was drawn into EDTA 5 %. Red cells were washed three times and resuspended in 2 % saline.

Tube test for titres. Agglutination was performed in glass tubes (10 x 75 mm) using 50 µL of the monoclonal antibody (ascitic fluid or its dilution in PBS solution containing 2 % BSA) and 50 µL of a 2 % saline-suspended red cells. The tubes were incubated at room temperature for 5 min, centrifuged at 500g for15 sec, and the bottom was gently resuspended. The titre was established as a maximal dilution offering a visible agglutination (1+).

Slide test for avidity and intensity. Avidity and intensity were established by mixing 50 µL of monoclonal antibody and 50 µL of 40 % red cell suspension on a slide. Avidity was estimated as the time required to give 1+ reaction. Intensity was evaluated from the agglutination clump size after 2 min (9).

Specificity. Specificity was established using previously described techniques for papainized group O red cell containing the following blood group antigens: Rh (C, c, E, e, C^w); Kell (K, k, kp^b, Js^a, Js^b), Lu (Lu^b), Duffy (Fya, Fyb); Kidd (jk^a, Jk^b); MNS (M, N, S, s); Lewis (Le^a, Le^b, Le^x); P (P_1).

The A_1(B) effect was studied by the tube test in 20 samples of red cells from group A_1 and 10 from A_2 previously treated with papain (10).

Evaluation of Mab as a blood-typing reagent. Ascitic was diluted in (1+ 3) PBS containing 2 % BSA and used for typing red cells from 300 non-selected donors. The results obtained by the three methods (slide, tube test and microplates) were compared with polyclonal antibodies in current use in our blood banks (9, 11).

RESULTS AND DISCUSSION

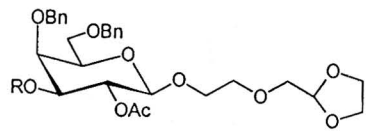
Synthesis of the trisaccharide

The trisaccharide specific for human B blood group is represented by the following chemical structure in which the central D-galactose residue is substituted at O-2 and O-3 by and α-L-fucose and α-D-galactose respectively. In the human A blood group, N-acetyl-α-D-galactosamine is located at the place of α-D-galactose. According to its use, the synthetic oligosaccharide should be provided with a spacer R to guarantee the covalent linkage with a macromolecular carrier or to a surface. Growing interest in synthetic human blood group substances is caused by their biomedical potential. Since the first synthesis (12)was accomplished, many laboratories have displayed their approaches (13-25). The differences between all these syntheses were to be found in the solution they offered to the most important problems associated not only with the synthesis but also with the spacer R, which allowed the linkage with the carrier through a covalent bond and maintained optimal distance for a better recognition.

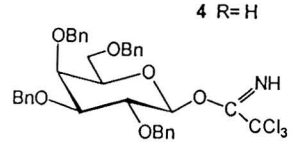
In this paper we present our approach to the synthesis of human blood group B trisaccharide, which includes a dioxolane type spacer we previously developed for synthetic oligosaccharides (26). The main feature of this spacer is its carbonyl function that is cryptic during the synthesis allowing a coupling by a reductive amination to the protein through their amino function.

Another important feature is the central galactose derivative. We used orthoester **2** prepared from acetobromogalactose **1** by its reaction first with methanol and collidine at reflux, then deacetylation and selective allylation at position 3 through the dibutyltin derivative and finally benzylation of hydroxyls 4 and 6.

The use of orthoester **2** as the key intermediate in the synthesis of human blood groups is noteworthy. The orthoester could be easily transformed (27) with acetyl bromide into the corresponding bromide which



3 R= All
4 R= H



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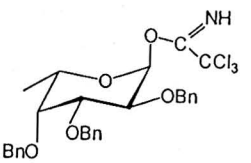

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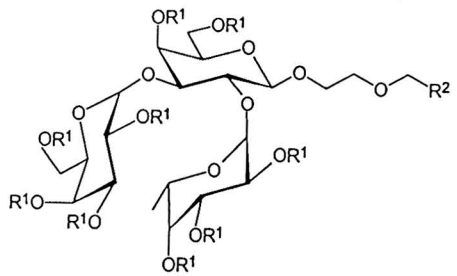

8 R^1= Bn; R^2= dioxolane
9 R^1= Ac; R^2= dioxolane
10 R^1= Ac; R^2= HCO
11 R^1= H; R^2= HCO

could be condensed with the spacer in the presence of mercuric bromide. The temporal protective groups at position 2 and 3 might be removed independently in order to introduce fucose or galactose. Since the former is more expensive, it is more convenient to provide first deallylation at position 3 giving the derivative **4** in a 32 % overall yield from **3**. The structure of **4** was confirmed by NMR spectroscopy. In the ^{13}C

spectra, the signal at 100.8 ppm is characteristic of C-1 with a β-D glycosidic linkage. Further confirmation was obtained from the doublet at 4.43 ppm in the ^{1}H NMR spectra. Its coupling constant J1-2 of 7.9 Hz is typical of a trans diaxial disposition for H1 and H2.

The introduction of α-D-galactose was accomplished using the trichloroacetimidate donor (28) **5** in diethyl ether in the presence of trimethylsilyl triflate. Under these conditions the α-linked disaccharide was the only product obtained and it was isolated in a 69 % yield.

The stereochemistry of the newly formed glycosidic bond in **7** was confirmed on the basis of the signal for those atoms located at the anomeric position. In the ^{1}H NMR spectra, the signal corresponding to H-1 is a doublet located at 4.95 ppm with a coupling constant J_{1-2} of 3.5Hz. In the heteronuclear correlation experiment, this signal is associated with the signal of C-1 located at 99.5 ppm in the ^{13}C spectra. Another important information was obtained from the signal of H-2, which is located at 4.39 ppm as a typical doublet of doublet confirming that this position is acetylated and no migration of this protective group took place during galactosylation.

The disaccharide **7** was deacetylated with sodium methoxide and fucosylated using the trichloroacetimidate **6** in the same conditions as for the introduction of **5**. Once again the 1,2-cis linked trisaccharide was the only product isolated.

From the H-H COSY experiment the signals corresponding to the three anomeric protons were readily distinguished at 5.58, 5.31 and 4.25 ppm with coupling constants J_{1-2} 3.3, 3.5Hz and 8.0Hz respectively. The two first correspond to the α-linked L-fucose and D-galactose; the last one, to the galactose linked with the spacer. The compound was acetylated after catalytic hydrogenolysis.

The generation of the aldehyde in the spacer is one of the most complex operations in this synthesis since glycosidic bonds, specially those of fucose, are acid sensitive. After intensive experimentation (26), we found that formic acid at room temperature for two hours splits off the dioxolane ring without affecting the glycosidic bonds.

This was confirmed by ^{1}H NMR spectroscopy which showed the signal of the aldehyde proton at 9.73 ppm, together with all the signals from the anomeric carbons at 103.8, 97.5 and 92.5 ppm. Free oligosaccharides were partially formulated making more complex reaction. follow-up. The use of peracetylated oligosaccharide for this reaction is recommended as a standard practice for the opening of the spacer. Careful deacetylation with 0.1 N MeONa in dry methanol gave the free activated trisaccharide that was immediately coupled with BSA for detection of antibodies or with KLH as a highly immunogenic vaccine for immunization.

Production and selection of monoclonal antibodies

The use of highly immunogenic hemocyanin (KLH) as a carrier gave a high anti-B titre in six of the ten immunized mice. Two fusions were performed with the mice with a higher anti-B titer. The results were reported in Table 1

All the hybridoma producing antibodies which recognized B trisaccharide coupled to BSA were cloned and retested. Those with a positive reaction were tested in slide tests for agglutination of red cells. Only three which showed a strong agglutination were chosen for further studies.

The molecular specificity of the three antibodies was studied by ELISA using the trisaccharide or its disaccharide fragment: Gal'-α-(1-3)-Gal' or Fuc-α-(1-2)-Gal' coupled to BSA. The first two (IgM) recognized also disaccharide Gal'(1-3)Gal' and thus are not specific for B-trisaccharide.

The third antibody (IgG) failed to recognize the disaccharide fragments. At least with these model compounds we can conclude that it is specific for human blood group B trisaccharide.

The affinity constant for antibody LAGS-B-03 was determined to be 5.28 + - 0.60 x $10^{10}(M^{-1})$.

Serological evaluation of Mab Lags-B-03

The specificity of monoclonal antibodies was usually more homogeneous for anti-B than for anti-A. However the preparation of a good anti-B reagent was more difficult (29) specially because of the low expression of B-antigen in A_1B and B-cordon cells.

A preliminary study of the three monoclonal agglutinating antibodies showed excellent agreement with their molecular specificity. Only the IgG (LAGS-B-03) was specific for B and AB red cells and was further studied.

Table 1
Results from the fusions

Fusion	No. hybrid	+ (B-BSA)	IgM	IgG	agglutination
1	384	34	12	22	1 IgM
2	318	35	10	25	1 IgG; 1 IgM

Table 2 shows that the antibody LAGS-B-03 strongly agglutinated all the red cells containing B-antigen. The titres were in the same order as reported by others (30) and independent of the B fenotype, thus exceeding the requirement for similar polyclonal antibodies. Avidity also exceeded the regulation for hemagglutinating reagents (29,31).

The presence of small quantities of B antigen in A_1 red cells was first demonstrated using anti-B monoclonal antibodies (32).

This loss of specificity in the A1-galactosaminyl transferase can be explained by its inappropriate *in vivo* transfer of small quantities of galactose to A_1 but not to A_2 red cells. This effect, known as A_1(B) phenomenon, was observed only for papainized red cells with a few very avid anti-B monoclonal antibodies (10).

As a rule all these antibodies were very potent and able to detect all B-subgroups. This phenomenon is also displayed by LAGS-B-03 antibody and therefore it could be classified as a very avid monoclonal anti-B. No B-subgroups(B3, Bv, Bint) were detected in our research. We believe that our antibody would detect these variants.

For blood typing reagents, the A_1(B) phenomenon may cause mistakes if the effect persists in a working dilution. In our case agglutination was observed only for undiluted ascites. It was very fragile and disappeared after the first dilution.

To further evaluate its usefulness as a blood-typing reagent, a preliminary study was performed with 300 unknown samples following slide, tube and microplate tests (A, 125; AB, 6; B, 44 and O, 125). The results demonstrated a complete correspondence with a routine polyclonal human antiserum.

Finally a more extended field trial was undertaken by several blood banks and demonstrated the usefulness of LAGS-B-03 as anti-B reagents.

In conclusion, our strategy represents an excellent alternative for the production of monoclonal antibodies with known molecular specificity and high affinity constant.

Table 2
Titres of MAb LAGS-B-03 versus red cells from different groups

	B n= 5	B cordon n= 4	A1B n= 5	A2B n= 3	A1 n= 4	A2 n= 3	O n= 5
Titres	256	128	256	256	0	0	0
Avidity(s)	2	2	3	3	-	-	-
Intensity	4+	4+	4+	-	-	-	-

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